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# Silencing of the Wnt transcription factor TCF4 sensitizes colorectal cancer cells to (chemo-) radiotherapy

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**A considerable percentage of rectal cancers are resistant to standard preoperative chemoradiotherapy. Because patients with a priori-resistant tumors do not benefit from multimodal treatment, understanding and overcoming this resistance remains of utmost clinical importance. We recently reported overexpression of the Wnt transcription factor TCF4, also known as TCF7L2, in rectal cancers that were resistant to 5-fluorouracil-based chemoradiotherapy. Because Wnt signaling has not been associated with treatment response, we aimed to investigate whether TCF4 mediates chemoradioresistance. RNA interference-mediated silencing of TCF4 was employed in three colorectal cancer (CRC) cell lines, and sensitivity to (chemo-) radiotherapy was assessed using a standard colony formation assay. Silencing of TCF4 caused a significant sensitization of CRC cells to clinically relevant doses of X-rays. This effect was restricted to tumor cells with high T cell factor (TCF) reporter activity, presumably in a β-catenin-independent manner. Radiosensitization was the consequence of (i) a transcriptional deregulation of Wnt/TCF4 target genes, (ii) a silencing-induced $G_2$/M phase arrest, (iii) an impaired ability to adequately halt cell cycle progression after radiation and (iv) a compromised DNA double strand break repair as assessed by γH2AX staining. Taken together, our results indicate a novel mechanism through which the Wnt transcription factor TCF4 mediates chemoradioresistance. Moreover, they suggest that TCF4 is a promising molecular target to sensitize resistant tumor cells to (chemo-) radiotherapy.**

## Introduction

The standard treatment for locally advanced rectal cancers consists of preoperative 5-fluorouracil (5-FU)-based chemoradiotherapy followed by radical surgery (1). This multimodal approach reduces local recurrence (2). However, clinical response to chemoradiotherapy varies greatly, and a considerable percentage of rectal cancers are chemoradioresistant, even if intensified regimens are being pursued (3). This represents a substantial clinical and socioeconomic problem. Thus, it is of utmost clinical importance to determine the molecular characteristics underlying this resistance and to identify effective strategies to overcome it (4). Previously, we have therefore used gene expression profiling of resistant and responsive rectal cancers from patients who had been treated with preoperative chemoradiotherapy within a phase III clinical trial (2) and found *TCF4* to be significantly overexpressed in resistant tumors (5).

**Abbreviations:** CRC, colorectal cancer; 5-FU, 5-fluorouracil; PCR, polymerase chain reaction; SCC, single-cell clone; TCF, T cell factor.

T cell factor 4 (TCF4), also known as TCF7L2, represents a key transcription factor that mediates canonical Wnt signaling, which plays a central role in embryonic development and in the maintenance of tissue homeostasis (6–8). Binding of Wnt ligands to cell surface receptors of the Frizzled family inhibits glycogen synthase kinase-3β-mediated phosphorylation of the cotranscription factor β-catenin, leading to its stabilization and subsequent accumulation in the nucleus. This results in binding to members of the TCF and lymphoid enhancer-binding factor family of transcription factors (9), which in turn induces or represses transcription of a plethora of target genes (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/).

Although aberrant Wnt signaling promotes colorectal cancer (CRC) development (6–8), it has not yet been associated with treatment resistance. In the present study, we therefore tested whether the observed overexpression of *TCF4* is of functional relevance for mediating chemoradioresistance in rectal cancer.

## Materials and methods

### *Cell culture*

Human CRC cell lines Caco-2, HT-29, SW1116, SW1463, SW480, SW620, SW837 and WiDr were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured as described recently (10). Cell line identity has been confirmed by short tandem repeat profiling (10), and absence of Mycoplasma contamination was tested periodically by polymerase chain reaction (PCR).

### *Establishment of stable single-cell clone populations*

Individual Expression Arrest™ lentiviral short-hairpin RNA constructs targeting *TCF4*, and a non-silencing control shRNA (shNeg), were obtained from Open Biosystems (Thermo Fisher Scientific, Huntsville, AL). The respective target sequences are listed in Supplementary Table S1, available at *Carcinogenesis* Online. As described recently (11), cells grown in log phase were transfected at 60–70% confluence with 2.5 μg of linearized vector DNA using the Amaxa Nucleofector System (Lonza, Cologne, Germany), and stable single-cell clone (SCC) populations were subsequently established.

### *Western blotting*

Cells were lysed in a lysis buffer containing 1% NP-40 and protease and phosphatase inhibitor cocktail. To separate cytosolic and nuclear fraction, cells were lysed using two separate lysis buffers containing 0.5% and 1% NP-40, respectively, and a protease and phosphatase inhibitor cocktail. Blocking was performed using 5% blotting grade milk. Membranes were probed overnight at 4°C with a rabbit anti-TCF4 antibody (1:10 000; Abcam, Cambridge, UK) or a mouse anti-β-catenin antibody (1:2000; Santa Cruz Biotechnology, Heidelberg, Germany) followed by a mouse anti-active-β-catenin antibody (1:2000; Millipore, Schwalbach, Germany). To confirm successful nuclear protein extraction, a rabbit anti-HDAC1 antibody was used (1:1000; New England Biolabs GmbH, Frankfurt am Main, Germany). Equal loading was ensured using a rabbit anti-actin antibody (1:2000; Sigma–Aldrich, Steinheim, Germany). As secondary antibody, either a goat anti-rabbit or a rabbit anti-mouse peroxidase linked antibody (both 1:30 000; Acris Antibodies, Herford, Germany) was used. Membranes were developed using an enhanced chemiluminescence detection system (ECL Advanced, GE Healthcare, Buckinghamshire, UK) and signals were detected using a CCD-Camera (LAS-3000 Imager; Fuji-Film, Düsseldorf, Germany). The optical density was measured using the ImageJ software (NIH).

### *Irradiation and determination of cell survival*

Tumor cells growing in log phase were seeded as single-cell suspensions into six-well plates and allowed to adhere overnight. Subsequently, cells were irradiated with a single dose of 1, 2, 4, 6 and 8 Gy of X-rays (Gulmay Medical Ltd, Camberley, UK), and a standard colony-forming assay was performed to determine the respective surviving fractions. After defined time periods (Supplementary Table S2 is available at *Carcinogenesis* Online), cells were fixed with 70% ethanol and stained. Colonies with >50 cells were scored as survivors. Non-irradiated cultures were used for data normalization. Experiments

were performed as technical triplicates and independently repeated three times. To estimate the sensitivity to chemoradiotherapy, cells were exposed to 3 μM of 5-FU (Sigma–Aldrich) for 16 h before irradiation, as described recently (10). Calculation of survival fractions (SF) was done using the equation SF = colonies counted/cells seeded × (plating efficiency/100). Survival variables α and β were fitted according to the linear quadratic equation. Dose-modifying factors at 37% survival and survival variables α and β have been calculated using KaleidaGraph (Synergy Software, Reading, PA) and are shown in Supplementary Table S3, available at *Carcinogenesis* Online.

### *Cell cycle analysis*

Cell cycle distribution was analyzed before, and 4 and 8 h after irradiation at 4 Gy. Cell membranes were permeabilized at −20°C overnight using 70% methanol. Subsequently, cells were treated with 10 μg/ml RNase A (Qiagen, Hilden, Germany) for 30 min at 37°C and stained with 20 μl/ml propidium iodide (Sigma–Aldrich) for 20 min at 37°C. DNA content was measured by flow cytometry (FACScan; BD Bioscience, Heidelberg, Germany) and analyzed using the FlowJo software package (Tree Star, Ashland, OR).

### *Immunofluorescence and quantification of phosphorylated histone H2AX foci formation*

Cells were seeded onto microscope slides and allowed to adhere overnight. Sixteen hours later, slides were irradiated at 2 Gy and fixed with 2% formaldehyde/phosphate-buffered saline for 15 min. Cells were permeabilized with 0.2% Triton X-100/phosphate-buffered saline/1% fetal bovine serum for 10 min on ice and blocked with 5% bovine serum albumin/1% fetal bovine serum/phosphate-buffered saline. Slides were incubated with a mouse anti-phosphohistone H2AX antibody (1:1000; Millipore) over night at 4°C, followed by incubation with a fluorescence-coupled mouse anti-rabbit secondary antibody (1:400; Alexa Fluor 594, Molecular Probes/Invitrogen, Darmstadt, Germany). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Sigma–Aldrich) and mounted using VECTASHIELD (Vector Laboratories, Peterborough, UK). Radiation-induced γH2AX foci were counted in at least 100 cells per sample using a fluorescence microscope (DM6000; Leica, Wetzlar, Germany) and the Leica Application Suite.

### *TOP-FLASH/FOP-FLASH dual luciferase reporter assay*

Cells were transfected with 100 ng TOP-FLASH plasmid containing six TCF-binding motifs (Millipore) or 100 ng FOP-FLASH control plasmid containing six-mutated TCF-binding motifs (Millipore). Each sample was cotransfected with 10 ng renilla luciferase plasmid (pRL-CMV; Promega, Mannheim, Germany) to normalize for cell viability and transfection efficiency. To estimate the inducibility of TCF reporter activity, cells were transfected with 100 ng of mutated β-catenin (S33Y; Millipore). This mutated protein cannot be inactivated by the degradation complex and translocates to the nucleus, where, after binding to TCFs, it leads to continuous reporter activity. Cell lysates were prepared using the Dual Luciferase Lysis Buffer (Promega), and luciferase activity was measured using a microplate reader (Mithras LB940; Berthold Technologies GmbH, Bad Wildbad, Germany). Promoter activity was calculated by dividing relative light units of specific TOP-FLASH and relative light units of non-specific FOP-FLASH.

### *Real-time PCR*

Real-time PCR was performed as described recently (10), and the corresponding primer sequences can be found in Supplementary Table S4, available at *Carcinogenesis* Online. The resulting cycle threshold (*C*t) values were normalized according to the mean of three housekeeping genes (i.e. *HPRT1*, *YWHAZ* and *GAPDH*) and the $2^{-\Delta\Delta CT}$ algorithm (12) was applied to analyze the relative changes in gene expression between two cell populations.

### *Statistical analysis*

A multiple linear regression model was used to describe the normalized surviving fraction as dependent variable, given the independent variables of irradiation dose, group (negative control versus SCC) and replicate pairing. A similar multiple linear regression was used to model the percentage of cells in $G_2$/M phase as dependent variable, given the independent variables of time-after-radiation, group (negative control versus SCC) and replicate pairing. An analysis of variance was performed on these models to reveal significant variables and interaction effects. For all other analyses, an unpaired two-tailed Student's *t*-test was used. *P*-values <0.05 were considered significant, and *P*-values <0.001 were considered highly significant. Data are expressed as mean ± standard error of the mean. All analyses were performed using the free statistical software R (version 2.9.2).

## Results

### *TCF4 expression correlates with chemoradioresistance in primary rectal cancers and in CRC cell lines*

Gene expression profiling of primary rectal cancers showed that the Wnt transcription factor *TCF4* was significantly overexpressed in those tumors that were resistant to preoperative 5-FU-based chemoradiotherapy (5). To test whether we can recapitulate these findings *in vitro*, we first measured TCF4 protein expression levels in CRC cell lines (Supplementary Figure S1A is available at *Carcinogenesis* Online). We then compared these expression levels with the respective *in vitro* sensitivities of these cell lines to 5-FU-based chemoradiotherapy, which we recently reported (10) and could confirm that elevated TCF4 expression was positively correlated with resistance to *in vitro* chemoradiotherapy (Supplementary Figure S1B is available at *Carcinogenesis* Online).

### *Silencing of TCF4 sensitizes CRC cell lines to irradiation*

To test whether the observed overexpression of *TCF4* is functionally relevant for treatment resistance, RNAi-mediated silencing was employed in three p53-mutant CRC cell lines (13,14) that expressed high levels of TCF4, i.e. SW837, HT-29 and SW480, using two short-hairpin RNA constructs. Stable SCC populations were established, and two SCCs from each vector were selected for further experimentation. Successful RNAi-mediated silencing of *TCF4* was demonstrated using western blot analysis (Figure 1A).

Subsequently, selected SCC populations were irradiated at clinically relevant doses of X-rays, and the respective surviving fractions were measured using a standard colony-forming assay. Compared with the non-silencing control shRNA, silencing of *TCF4* significantly increased sensitivity of all SW837 and SW480 SCCs to radiation ($P < 10^{-16}$ and $P < 10^{-16}$, respectively; multiple linear regression model). In clear contrast, *TCF4* silencing had no effect on HT-29 cells ($P = 0.7$; Figure 1B, Supplementary Figure S2 is available at *Carcinogenesis* Online). Survival variables α and β and dose modulation factors are shown in Supplementary Table S3, available at *Carcinogenesis* Online. Notably, in SW837 and SW480, TCF4 protein expression levels increased in response to treatment with 6 Gy of X-rays, whereas the expression of TCF4 was unchanged in HT-29 (Supplementary Figure S3 is available at *Carcinogenesis* Online).

Because the standard therapy for locally advanced rectal cancers comprises 5-FU-based chemoradiotherapy, we also employed a combination of 3 μM of 5-FU and irradiation, as recently described (10) and observed very similar results. RNAi against *TCF4* sensitized SW837 SCCs, but not HT-29 SCCs (Supplementary Figure S4 is available at *Carcinogenesis* Online).

### *Silencing of TCF4 induces accumulation of SW837 cells in $G_2$/M phase*

Next, we investigated the cellular processes leading to the significant radiosensitization of SW837 SCCs. These and other follow-up experiments were performed exclusively in SW837 and HT-29. These two lines were chosen because both are highly chemoradioresistant, and we wanted to analyze the phenotype of resistance and sensitization, respectively.

Cells are not equally sensitive to radiation throughout the cell cycle but show increased radiation sensitivity in $G_2$/M (15). We therefore performed cell cycle analyses of unirradiated SW837 and HT-29 SCC populations (exemplified in Figure 2A). Regarding SW837, an average of 23% of cells from different SCCs were captured in the $G_2$/M phase compared with only 15% of cells from the non-silencing control (Figure 2B). This difference was statistically significant ($P < 0.01$) and suggests that radiosensitization of SW837 SCCs is at least in part attributable to an accumulation of cells in radiosensitive phases of the cell cycle (15). In clear contrast, no significant changes in cell cycle distribution were detected between HT-29 SCCs and the respective non-silencing control (Figure 2A and B).

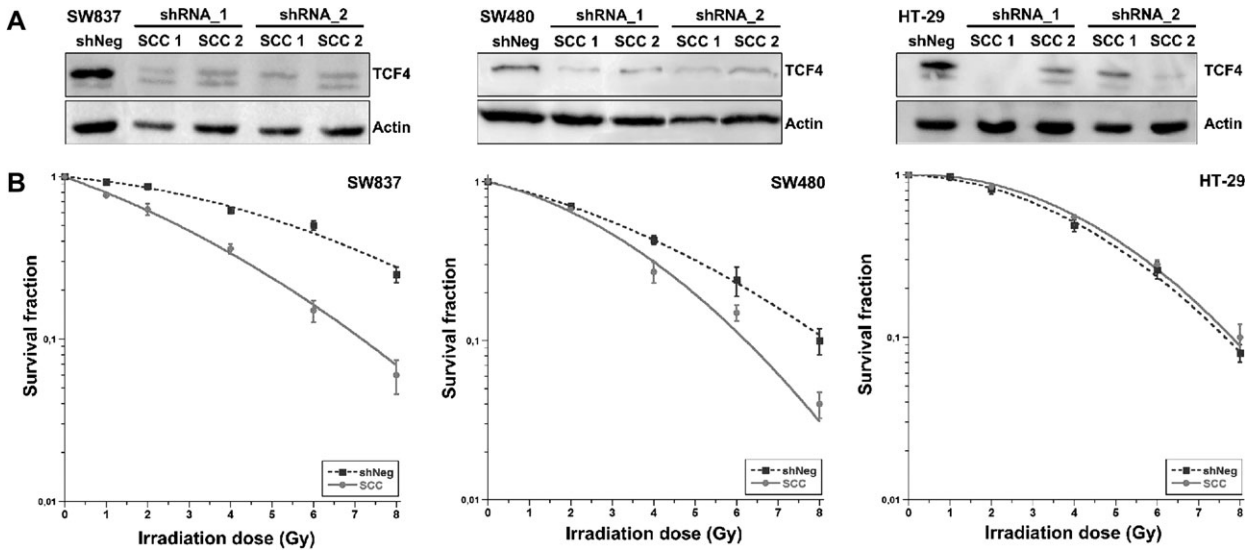


**Fig. 1.** Silencing of *TCF4* sensitizes SW837 and SW480 to radiation. (**A**) Cell lines were transfected with two individual shRNA constructs targeting *TCF4* and stable SCC populations were established. Compared with a non-silencing control shRNA (shNeg), all clones exhibited markedly reduced protein levels of TCF4. (**B**) Selected SCCs were irradiated at clinically relevant doses of X-rays. A standard colony-forming assay demonstrated that silencing of *TCF4* significantly increased the radiosensitivity of SW480 and SW837 but not of HT-29 (exemplified for one representative SCC per cell line; see also Supplementary Figure S2, available at *Carcinogenesis* Online). Data are presented as mean of three independent experiments ± standard error of the mean.

*Silencing of TCF4 impairs radiation-induced $G_2$/M arrest in SW837*

Radiation exposure of eukaryotic cells results in a cell cycle delay required for DNA damage repair or induction of apoptosis (16). We therefore tested whether silencing of *TCF4* leads to impaired cell cycle control after radiation as reflected by a lack of accumulation in $G_2$/M phase. In the SW837 non-silencing control, the fraction of cells in $G_2$/M phase markedly increased from 15% at the time of radiation to 36% 8 h after radiation (Figure 2C). In contrast, we only observed a slight increase in SW837 SCCs from 23 to 30% (Figure 2C). This difference in cell cycle control, i.e. changes in the cell cycle distribution over time, was statistically significant ($P < 0.05$) between the negative control and the respective SCC populations in SW837. In HT-29, however, silencing of *TCF4* did not alter cell cycle progression, i.e. both populations (SCCs and control) adequately arrested in $G_2$/M phase 8 h after radiation (Figure 2D), corresponding to a lack of radiosensitization.

*Silencing of TCF4 impairs DNA double strand break repair in SW837*

Insufficient DNA damage repair is an important component of radiation-induced cell killing (17,18). To determine whether the radiosensitization following RNAi against *TCF4* is indeed attributable to impaired DNA damage repair, we monitored the presence and persistence of phosphorylated histone H2AX (γH2AX) foci (17,18).

Regarding SW837, both populations (shNeg and SCCs) showed very few γH2AX foci in unirradiated cells and comparable levels of foci induction 15 min after irradiation at 2 Gy (exemplified in Figure 3A). Importantly, however, 24 h after irradiation, few foci remained in the non-silencing control, whereas the number of γH2AX foci in SW837 SCCs persisted at considerably higher levels (Figure 3A). This difference was statistically highly significant ($P < 0.001$; Figure 3B). These foci can be considered 'residual', pointing to incomplete DNA double strand break (DSB) repair, which results in radiosensitivity (19,20).

With respect to HT-29, both populations (shNeg and SCCs) showed minimal γH2AX foci in unirradiated cells and similar induction of foci 15 min after irradiation at 2 Gy (exemplified in Figure 3C). In striking contrast to SW837, both HT-29 populations exhibited an equally low number of γH2AX foci 24 h after irradiation (Figure 3C and D). These data support the notion that silencing of *TCF4* leads to a significant impairment of DNA DSB repair in SW837, but not in HT-29.

*TCF reporter activity determines radiosensitization in a β-catenin independent manner*

TCF4 is a key transcription factor of canonical Wnt signaling (6–9). We therefore speculated that, despite comparable baseline protein expression levels of TCF4 in SW837 and HT-29, differences in the transcriptional activity might have caused the heterogeneous sensitivity of these cell lines to irradiation upon exposure to RNAi against *TCF4*. Using the TOP-FLASH/FOP-FLASH reporter assay, we established higher basal reporter activity for wild-type SW837 cells (TOP/FOP: 6.9) than for wild-type HT-29 cells (TOP/FOP: 1.6). This difference was statistically significant ($P < 0.01$; Figure 4A).

TCF4 is a binding partner of β-catenin, as both mediate the effects of canonical Wnt signaling. Notably, overexpression of mutated β-catenin (S33Y), which activates TCFs but cannot be inactivated, caused a >10-fold higher increase in reporter activity in wild-type SW837 cells (TOP/FOP: 34.3) than in wild-type HT-29 cells (TOP/FOP: 2.9; $P < 0.001$).

Next, we analyzed the nuclear and cytosolic levels of β-catenin. Wild-type SW837 and HT-29 cells exhibited similar protein levels of both nuclear and cytosolic β-catenin (Figure 4B). These results suggest that radiosensitization following silencing of *TCF4* is determined by TCF transcriptional activity but independent of β-catenin activity.

*Deregulation of Wnt/TCF4 signaling in SW837*

Finally, to demonstrate that silencing of *TCF4* results in a transcriptional deregulation of Wnt/TCF4 signaling in SW837, but not in HT-29, we measured the expression levels of selected target genes (21–23). Using real-time PCR, we observed a considerable deregulation of *CCND1*, *DKK1* and *MYC* in SW837 (Figure 5), with average fold-changes of 2.4 (*CCND1*, upregulated following silencing of *TCF4*), 18.4 (*DKK1*, downregulated) and 2.7 (*MYC*, downregulated). Notably, in HT-29, these downstream target genes either showed no prominent deregulation (*CCND1*; Figure 5) or their deregulation was inconsistent compared with SW837 (*DKK* and *MYC*; Figure 5). Collectively, these results demonstrate that RNAi against *TCF4* resulted in a transcriptional deregulation of Wnt/TCF4 signaling in SW837, which was

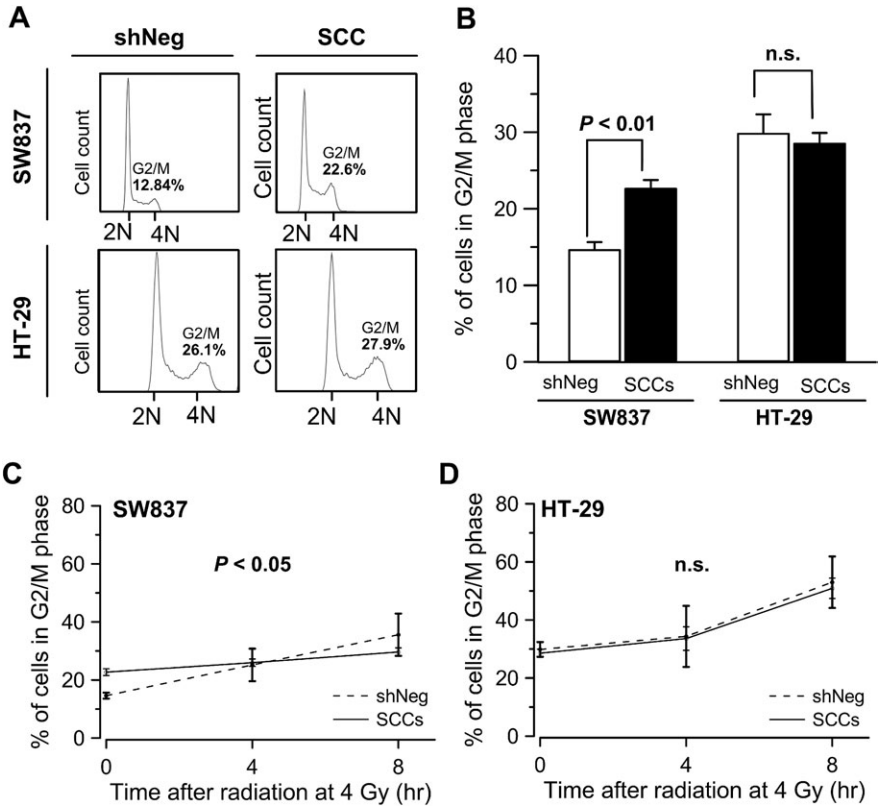


**Fig. 2.** Silencing of *TCF4* in SW837 induces a $G_2$/M arrest and impairs cell cycle control after radiation. (**A**) Representative cell cycle analysis of unirradiated tumor cells. (**B**) Compared with the non-silencing control, unirradiated SW837 SCCs exhibited a significantly higher fraction of cells in the radiosensitive $G_2$/M phase ($P < 0.01$), whereas silencing of *TCF4* in HT-29 did not alter cell cycle distribution. There was no difference in cell cycle distribution/progression between unirradiated shTCF4 SCCs and the respective non-silencing controls (data not shown). (**C**) Cell cycle analyses were also performed after irradiation. In the SW837 non-silencing control, the fraction of cells in $G_2$/M phase significantly increased 4 and 8 h after irradiation at 4 Gy ($P < 0.05$). In contrast, there was only a slight increase in SW837 SCCs, indicating an impaired ability of these SCCs to adequately halt cell cycle progression after irradiation. (**D**) Irradiation of both non-silencing control and SCCs in HT-29 did not alter cell cycle progression. Data are presented as mean of at least three independent experiments ± standard error of the mean.

associated with a significant sensitization to clinically relevant doses of X-rays.

## Discussion

Resistance to preoperative chemoradiotherapy represents a major clinical problem in the treatment of rectal cancer. Consequently, the identification of novel therapeutic targets whose modification could be harnessed to sensitize a priori-resistant tumors to radiation is exceedingly important. Our earlier studies showed that the Wnt transcription factor *TCF4* was overexpressed in primary rectal cancers that were resistant to preoperative chemoradiotherapy (5). The fact that Wnt signaling has not been previously associated with treatment resistance prompted us to explore whether this finding is functionally relevant. We now report that the Wnt transcription factor TCF4 mediates resistance to treatment with (chemo-) radiotherapy.

We first observed that TCF4 expression levels also correlated with (chemo-) radioresistance in CRC cell lines (Supplementary Figure S1B is available at *Carcinogenesis* Online). In order to explore the mechanistic basis of this correlation, we silenced *TCF4* using RNA interference and measured the phenotypic effects. Indeed, silencing of *TCF4* considerably increased sensitivity of SW480 and SW837 to clinically relevant doses of X-rays (Figure 1B, Supplementary Figure S2 is available at *Carcinogenesis* Online). However, this response was not uniform: the cell line HT-29 remained at resistance levels observed before silencing *TCF4*. A similar divergence became apparent when wild-type cell lines were treated with irradiation. In SW837 and SW480, TCF4 protein expression levels increased in response to treatment with 6 Gy of X-rays, whereas the expression of TCF4 was unchanged in HT-29 (Supplementary Figure S3 is available at *Carcinogenesis* Online). This suggests that TCF4 plays different roles in these cell lines in mediating the response to irradiation.

To further clarify the functional mechanism of this increased sensitivity of SW837 after TCF4 depletion, we performed cell cycle measurements. Silencing of *TCF4* was paralleled by an increased fraction of cells in the $G_2$/M phase of the cell cycle at the time of irradiation (0 h; Figure 2A and B). This phase is known for increased vulnerability to radiation-induced DNA damage (15). Of note, there was no change in cell cycle distribution upon silencing of *TCF4* in HT-29, entirely consistent with its unchanged radiation response.

Exposure of cells to radiation *per se* leads to an arrest of cells in $G_2$/M phase, allowing time for DNA damage repair (16). In SW837, however, the $G_2$/M arrest after irradiation (4 and 8 h) was significantly less pronounced after silencing of *TCF4* (Figure 2C). This impaired ability of the SW837 SCCs to halt cell cycle progression after irradiation was associated with a high number of persisting γH2AX foci (Figure 3A and B), which accumulate at sites of unrepaired DNA (17). These residual foci in SW837 SCCs suggest a compromised DNA DSB repair as an explanation for the increased radiosensitivity (19,20). In other words, these SCCs may re-enter the cell cycle with persistent DNA DSBs, which would be consistent with an increased radiosensitivity (24,25). In contrast, radiation-induced cell cycle arrest was not affected by silencing of *TCF4* in HT-29 (Figure 2D), and the amount of γH2AX foci returned to near baseline levels after 24 h

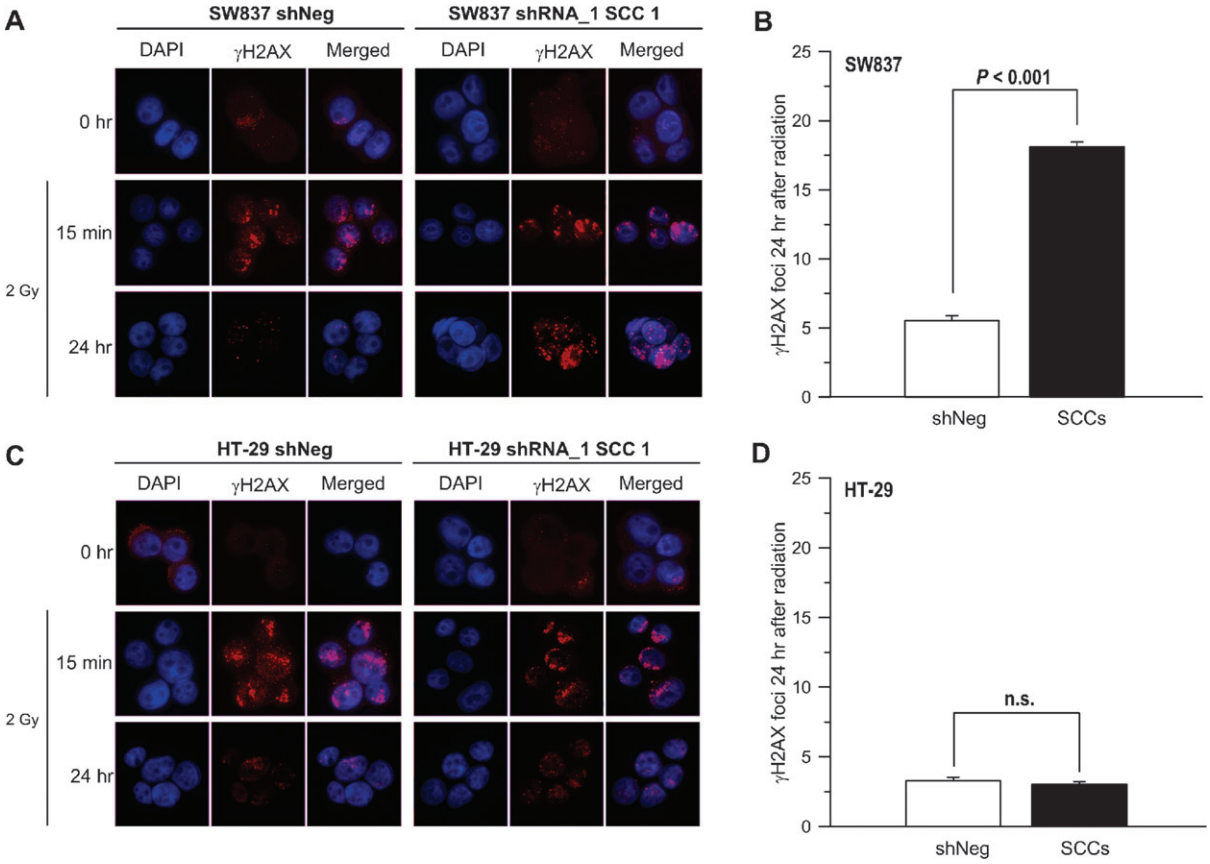


**Fig. 3.** Impaired radiation-induced DNA DSB repair in SW837 as indicator of increased radiosensitivity. (**A**) Representative experiment for SW837. Both populations (negative control and SCC) showed very few phosphorylated histone H2AX (γH2AX) foci at the time of radiation and comparable induction of foci 15 min after irradiation at 2 Gy. Twenty-four hours after irradiation, multiple γH2AX foci were present in the SCCs, whereas considerably fewer foci remained in the non-silencing control. (**B**) On average, there were highly significantly more γH2AX foci in SW837 SCCs 24 h after irradiation compared with shNeg ($P < 0.001$). (**C**) Representative experiment for HT-29. Both populations (negative control and SCC) showed very few γH2AX foci at the time of radiation, and exhibited a comparable induction of foci 15 min and 24 h after after irradiation at 2 Gy. (D) In stark contrast to SW837, there was a similar level of γH2AX foci in negative control and HT-29 SCCs 24 h after irradiation. Data are presented as mean of three independent experiments ± standard error of the mean.

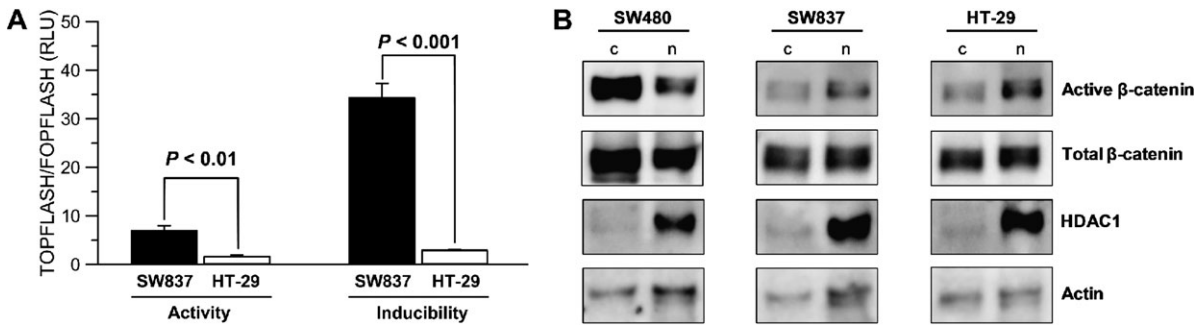


**Fig. 4.** Transcriptional activity of TCF determines radioresistance in SW837 in a β-catenin independent manner. (**A**) Normalized luciferase measurements. We established higher basal TCF reporter activity for wild-type SW837 cells (specific TOP-FLASH over non-specific FOP-FLASH activity: 6.9) than for wild-type HT-29 cells (TOP/FOP: 1.6; $P < 0.01$). Overexpression of mutated β-catenin (S33Y), which binds to TCFs and cannot be inactivated, caused a >10-fold higher increase in reporter activity in SW837 (TOP/FOP: 34.3) than HT-29 (TOP/FOP: 2.9; $P < 0.001$). Data are presented as mean of three independent experiments ± standard error of the mean. (**B**) Based on western blot analysis, SW837 and HT-29 show comparable nuclear and cytosolic expression levels of both active (phosphorylated) and total β-catenin.

regardless of the presence of TCF4 (Figure 3C and D). Taken together, TCF4 seems to play an important role in regulating both cell cycle and DNA damage repair in a subset of CRC cells.

It is important to note that although connections between Wnt signaling and cell cycle regulation have been postulated (26,27), only limited mechanistic details in support of the interplay between the two processes have been reported (26,27). Interestingly, van de Wetering *et al.* (28) previously observed that inhibition of TCF4 activity in LS174T (wild-type p53 protein) and in DLD-1 (mutant p53 protein) using overexpression of a dominant-negative TCF4 protein mediated a $G_1$ arrest. This discrepancy with our observations could have been because of two reasons. Firstly, these authors have used a method that completely diminishes TCF4 protein levels, whereas, in our experiments, up to 10–20% of TCF4 protein remained following RNAi

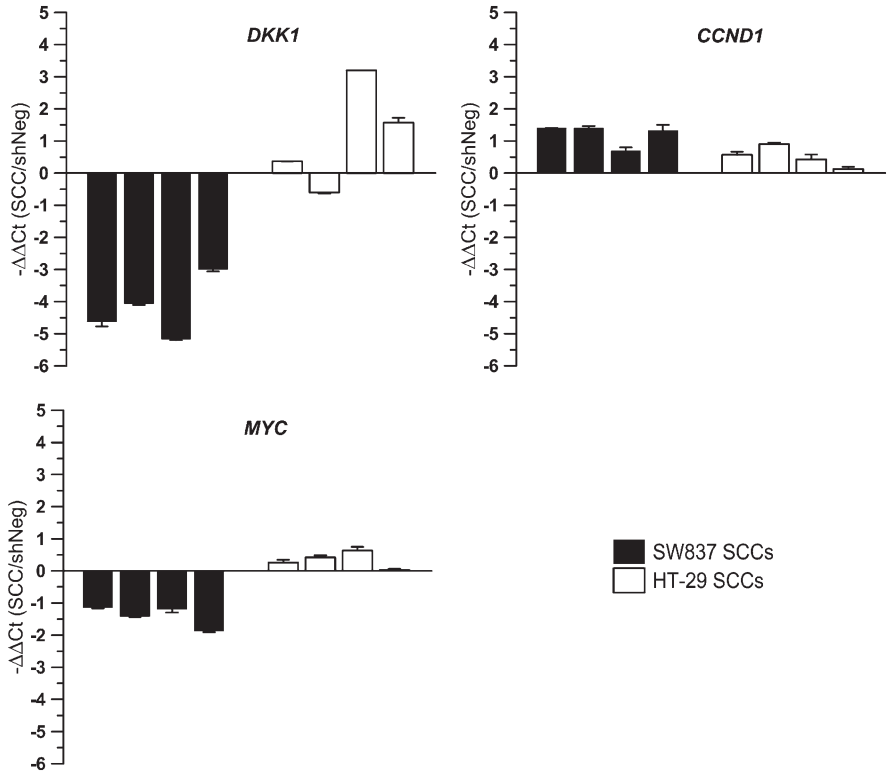


**Fig. 5.** Silencing of *TCF4* results in a transcriptional deregulation of Wnt/TCF4 signaling in SW837. Silencing of *TCF4* in SW837 (black) leads to a transcriptional deregulation of the Wnt/TCF4 target genes *MYC* (downregulated, average fold-change of 2.7), *Cyclin D1* (upregulated, average fold-change of 2.4) and *DKK1* (downregulated, average fold-change of 18.4), whereas neither a prominent or consistent deregulation was observed in HT-29 (white). Plotted are the normalized expression levels of *TCF4* in four SSCs relative to the negative control shNeg ($-\Delta\Delta Ct$). Data are presented as mean ± standard error of the mean.

exposure (Figure 1A). Secondly, van de Wetering *et al.* analyzed CRC cell lines that were mismatch repair deficient. Nevertheless, our observation that RNAi against *TCF4* resulted in significant changes in cell cycle distribution and an impaired ability to adequately halt cell cycle progression in response to irradiation adds weight to the growing body of evidence that the cell cycle machinery and Wnt signaling are functionally linked.

TCF4 represents a key downstream effector of canonical Wnt signaling (6–8). We therefore speculated that the diverse responses of SW837 and HT-29 may depend on varying transcriptional activities of TCF4. Indeed, unstimulated baseline levels of TCF reporter activity were higher in SW837 compared with HT-29 (Figure 4A). Moreover, reporter activity in SW837 increased dramatically upon overexpression of a mutated β-catenin protein (S33Y), which leads to constitutive TCF/β-catenin activity, while there was only a slight increase in HT-29 (Figure 4A). At first glance, these results would indicate that SW837 is much more responsive to β-catenin binding than HT-29. Interestingly, however, we observed comparable nuclear and cytosolic levels of β-catenin in SW837 and HT-29 (Figure 4B), suggesting that the observed effect is β-catenin independent. In this context, recent observations demonstrate that lymphoid enhancer-binding factor/TCF family members do not exclusively confer canonical Wnt/β-catenin signaling but also function as transcription factors in an alternative, β-catenin independent manner (29–32), and, presumably, in β-catenin-independent Wnt signaling (33–35).

Finally, we observed a transcriptional deregulation of the Wnt/TCF4 target genes *CCND1*, *DKK1* and *MYC* in SW837 SCCs, but not in HT-29 SCCs (Figure 5). Surprisingly, *DKK1* expression decreased upon silencing of *TCF4* in SW837 (Figure 5). At a first glance, this seems counterintuitive because *DKK1*, a putative Wnt antagonist, represents a tumor suppressor gene (36). The same holds true for the observed upregulation of CCDN1. Cyclin D1 represents a core component of the cell cycle machinery (37,38) and has been very recently implicated in DNA repair (39). Considering the increased fraction of SW837 cells in $G_2$/M phase following RNAi against *TCF4*, this upregulation is unexpected and may be attributed to the fact that stable SCC populations have been used in this study. These clones have been cultured for longer periods of time, potentially allowing time to counterbalance certain consequences of diminished expression of the transcription factor TCF4. Nevertheless, the transcriptional deregulation of Wnt signaling, irrespective of the directionality of deregulation, is consistent with the prevailing interpretation that disturbing the net cellular homeostasis of the Wnt pathway constitutes a critical step toward a tumor promoting function (40).

Although we are the first to report that inhibition of Wnt/TCF4 signaling sensitizes CRC cell lines to radiation and chemoradiotherapy, preliminary evidence from other model systems is consistent with these findings. Firstly, our own group recently reported that Wnt signaling pathway genes were significantly over-represented within a gene expression signature for *in vitro* sensitivity of CRC cells lines to 5-FU-based chemoradiotherapy (10). Secondly, based on the fact that Wnt signaling has been implicated in regulating the behavior of both somatic stem cells and tumor-initiating 'cancer stem' cells (41,42), other authors demonstrated that Wnt signaling mediates radiation resistance of mammary progenitor cells in mice (43,44). Thirdly, Flahaut *et al.* (45) reported that the frizzled-1 Wnt receptor FZD1 mediates chemoresistance in neuroblastoma cell lines through MDR1, whereas Shou *et al.* observed that overexpression of *DKK1* sensitized brain tumor cells to apoptosis upon treatment with DNA-alkylating agents (46). And fourthly, Kriegl *et al.* (47) recently demonstrated that TCF4 expression, based on immunohistochemical analyses of primary CRCs, was a negative prognostic factor associated with shorter overall survival.

In summary, we provide the first evidence that the Wnt transcription factor TCF4 is intricately involved in mediating resistance of CRC cell lines and primary rectal cancers to radiation and

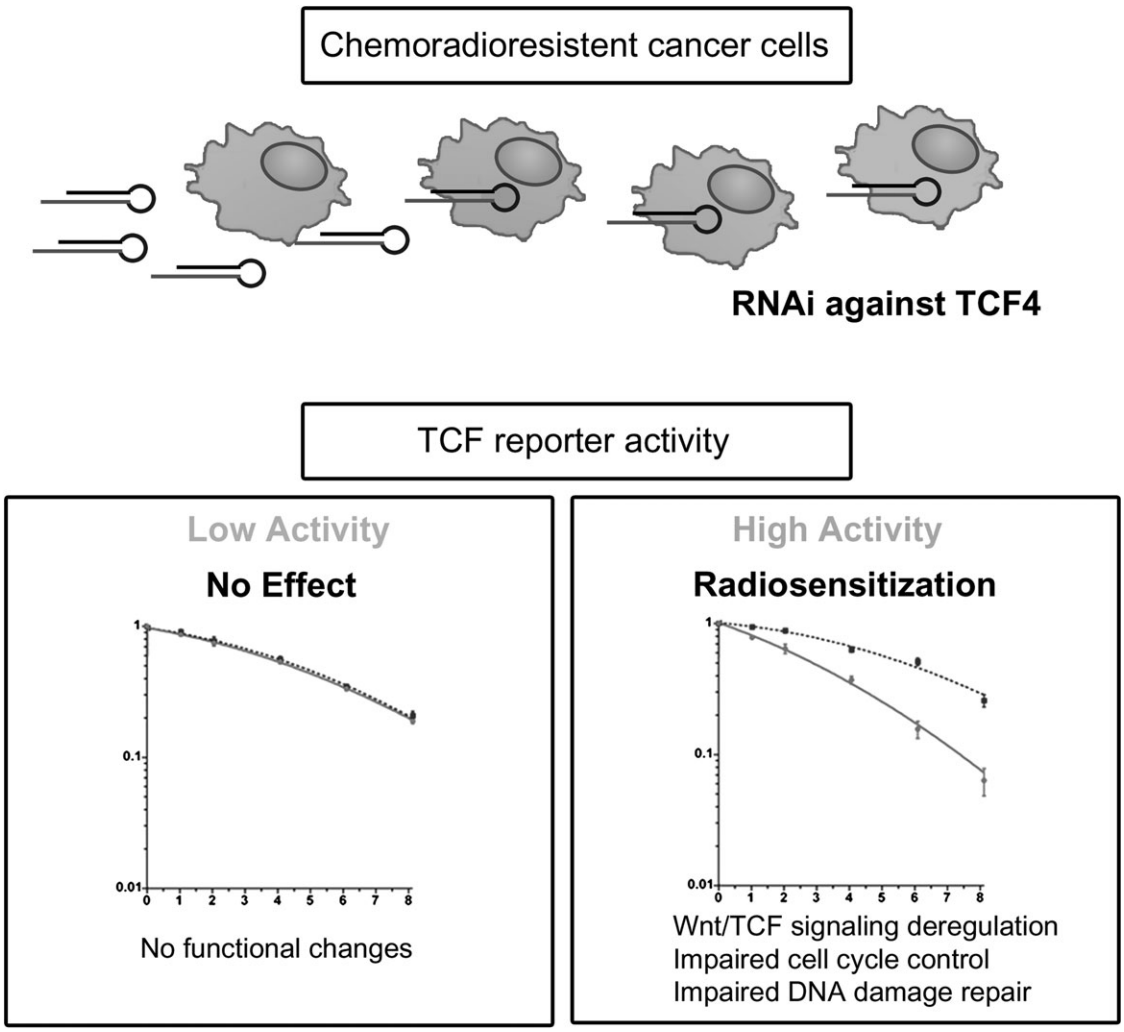


**Fig. 6.** Mechanistic model for the novel role of Wnt/TCF4 signaling. Mechanistic model depicting a novel mechanism through which the Wnt transcription factor TCF4 mediates resistance of CRC cells to chemoradiotherapy.

chemoradiotherapy. TCF4 therefore represents a promising molecular target to sensitize a priori-resistant rectal cancers to irradiation. Although we began to decipher the underlying cellular mechanisms (Figure 6), future studies will ultimately elucidate the downstream effects and regulation of TCF4 that mediate this important phenomenon, and they will further characterize the putative β-catenin independency of this novel role of TCF4. If further validated, Wnt/TCF4 signaling inhibition may represent an effective strategy to increase the fraction of patients that respond to multimodal treatment and improve overall survival.

## Supplementary material

Supplementary Tables S1–S4 and Figures S1–S4 can be found at http://carcin.oxfordjournals.org/

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